

# CmlI N-Oxygenase Catalyzes the Final Three Steps in Chloramphenicol Biosynthesis without Dissociation of Intermediates

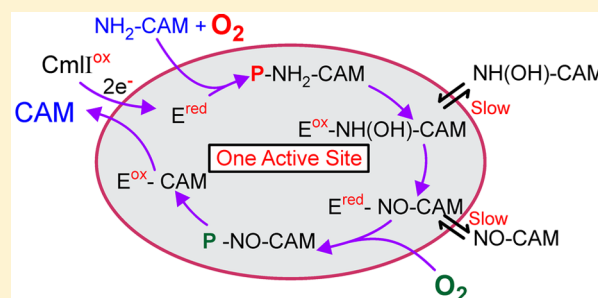
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**S** Supporting Information

**ABSTRACT:** CmlI catalyzes the six-electron oxidation of an aryl-amine precursor (NH<sub>2</sub>-CAM) to the aryl-nitro group of chloramphenicol (CAM). The active site of CmlI contains a (hydr)oxo- and carboxylate-bridged dinuclear iron cluster. During catalysis, a novel diferric-peroxo intermediate **P** is formed and is thought to directly effect oxygenase chemistry. Peroxo intermediates can facilitate at most two-electron oxidations, so the biosynthetic pathway of CmlI must involve at least three steps. Here, kinetic techniques are used to characterize the rate and/or dissociation constants for each step by taking advantage of the remarkable stability of **P** in the absence of substrates (decay  $t_{1/2} = 3$  h at 4 °C) and the visible chromophore of the diiron cluster. It is found that diferrous CmlI (CmlI<sup>red</sup>) can react with NH<sub>2</sub>-CAM and O<sub>2</sub> in either order to form a P-NH<sub>2</sub>-CAM intermediate. P-NH<sub>2</sub>-CAM undergoes rapid oxygen transfer to form a diferric CmlI (CmlI<sup>ox</sup>) complex with the aryl-hydroxylamine [NH(OH)-CAM] pathway intermediate. CmlI<sup>ox</sup>-NH(OH)-CAM undergoes a rapid internal redox reaction to form a CmlI<sup>red</sup>-nitroso-CAM (NO-CAM) complex. O<sub>2</sub> binding results in formation of P-NO-CAM that converts to CmlI<sup>ox</sup>-CAM by enzyme-mediated oxygen atom transfer. The kinetic analysis indicates that there is little dissociation of pathway intermediates as the reaction progresses. Reactions initiated by adding pathway intermediates from solution occur much more slowly than those in which the intermediate is generated in the active site as part of the catalytic process. Thus, CmlI is able to preserve efficiency and specificity while avoiding adventitious chemistry by performing the entire six-electron oxidation in one active site.



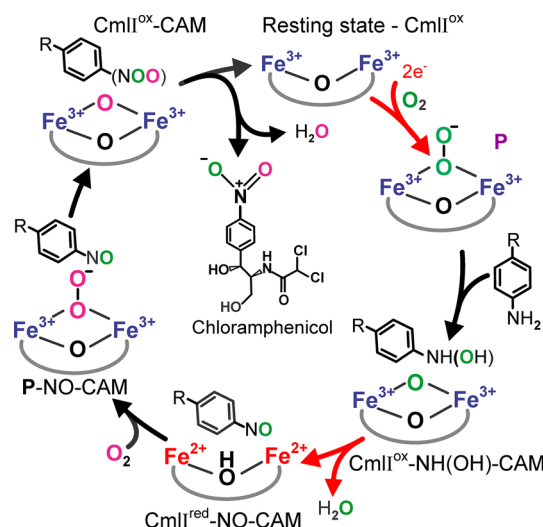
The final steps in the biosynthesis of chloramphenicol [CAM (Figure 1)] by *Streptomyces venezuelae* are catalyzed by the N-oxygenase CmlI.<sup>1,2</sup> The substrate for CmlI is the aryl-amine analogue of CAM [D-threo-1-(4-amino-phenyl)-2-dichloroacetyl-amino-1,3-propanediol or NH<sub>2</sub>-CAM], which undergoes a six-electron oxidation, including two oxygenation steps, to yield the aryl-nitro group of the active antibiotic (Figure 1). Spectroscopic studies and the X-ray crystal structure of CmlI have shown that it has a (hydr)oxo- and carboxylate-bridged dinuclear iron cluster in the active site.<sup>2,3</sup> Structurally similar clusters are found in many enzymes that activate O<sub>2</sub> and then catalyze reactions such as functionalization of unactivated C–H bonds, desaturation reactions, aromatic hydroxylation, and radical formation, but, notably, not aryl-amine oxygenation.<sup>4–14</sup> Conversely, CmlI and its structural and function homologue, AurF,<sup>15–19</sup> do not catalyze the types of reactions common to other diiron oxygenases. Kinetic and spectroscopic studies of CmlI and AurF have shown that, despite the structural similarities to other diiron oxygenases, a different type of reactive oxygen intermediate is generated during catalysis. Both N-oxygenase enzymes generate long-lived peroxo intermediates that react

directly with substrates,<sup>2,18</sup> as opposed to the high-valence oxo intermediates often formed by the other types of diiron oxygen-activating enzymes.<sup>5,20,21</sup> In CmlI, the peroxo intermediate (**P**) is exceptionally stable (decay  $t_{1/2} = 3$  h at 4 °C and pH 9) when it is generated by adding O<sub>2</sub> to the diferrous enzyme (CmlI<sup>red</sup>) in the absence of substrates.<sup>2</sup> When **P** is mixed with aryl-amine substrates, it reacts in milliseconds to yield oxygenated products. The stability of **P** greatly facilitates transient kinetic experiments designed to investigate the kinetics of its reactivity with substrates. In recent studies, we have shown that it will react with the likely intermediates of the chloramphenicol biosynthetic pathway, including aryl-nitroso-CAM (NO-CAM), which has electronic properties different from those of NH<sub>2</sub>-CAM, demonstrating the catalytic diversity of **P**.<sup>22</sup> Spectroscopic studies have also shown that **P** has a peroxo-bridging structure significantly different from the bridging *cis*-μ-1,2-peroxo structure found for almost all other characterized diiron-peroxo intermediates.<sup>2,23</sup>

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**Figure 1.** Proposed mechanistic cycle for N-oxygenation by CmlI.<sup>22,23</sup> Diferrous cluster reduction steps are shown with red arrows. The color coding of the oxygen atoms highlights the fact that oxygen atoms from two different O<sub>2</sub> molecules are incorporated into the final product. R = 2-(dichloroacetyl-amino)-1,3-propanediol.

Peroxo intermediates, like the high-valence iron-oxo intermediates of other diiron oxygenases,<sup>5,21,24,25</sup> can perform at most a two-electron oxidation reaction, and thus, the biosynthetic pathway of CmlI must have at least three steps. Studies of both CmlI and AurF<sup>19,22</sup> have defined mechanistic proposals that differ substantially from the most straightforward scheme of simply repeating the oxygenation reaction of P three times with three pathway intermediates, a process that would require six external electrons.<sup>16,26</sup> The iteration of this mechanism that we have proposed for CmlI is shown in Figure 1.<sup>22,23</sup> In this scheme, P is formed and reacted with NH<sub>2</sub>-CAM to make diferrous CmlI (CmlI<sup>red</sup>) and an aryl-hydroxylamino-CAM [NH(OH)-CAM] intermediate. The resulting CmlI<sup>ox</sup>-NH(OH)-CAM complex undergoes an internal redox reaction to yield CmlI<sup>red</sup>-NO-CAM that can bind O<sub>2</sub> to re-form P as a P-NO-CAM complex. The final oxygenation reaction by this complex yields CAM and CmlI<sup>ox</sup>. The internal reduction step means that this mechanism requires only two external electrons that are used to form the first P. This mechanism is supported by stepwise product formation studies showing the generation and reactivity of the predicted intermediates.<sup>22</sup> Also, it has been observed that forming P with one isotope of O<sub>2</sub> and then performing the reaction in a vessel with a different isotope of O<sub>2</sub> in the headspace results in a mixed isotope nitro group in CAM, thereby demonstrating re-formation of P as part of the biosynthetic pathway.<sup>22</sup>

In the studies reported here, the dynamics of the predicted reaction pathway of CmlI are studied using transient kinetic techniques to determine rate and/or dissociation constants for each step in the process. In this way, the main tenets of the mechanism, including the internal reduction step and the reaction of P with the chemically distinct substituent groups of the pathway intermediates, can be independently demonstrated and quantified. In the course of this study, more subtle aspects of the process have also come to light, including the mechanism by which the enzyme catalyzes the multistep reaction without a loss of intermediates and with perfect specificity.

## EXPERIMENTAL PROCEDURES

Standard materials and procedures are described in the [Supporting Information](#). The D-threo-1-(4-aminophenyl)-2-dichloroacetyl-amino-1,3-propanediol (NH<sub>2</sub>-CAM) substrate was obtained from Toronto Research Chemicals (Toronto, ON). The NH(OH)-CAM and NO-CAM substrates were synthesized as previously described.<sup>22</sup>

**Stopped-Flow Analysis of P Formation and Single-Turnover Reactions.** Stopped-flow reactions were performed using an Applied Photophysics model SX.18MV stopped-flow device configured for single-wavelength collection at 300 nm (P formation reaction), 390 nm [reactions with NH(OH)-CAM], or 480 nm (reactions with NH<sub>2</sub>-CAM or NO-CAM). All reactions were performed in 50 mM Bicine (pH 9) at 4 °C. CmlI is fully functional between pH 6 and 9, but the stability of P is greatly enhanced at high pH. Consequently, it is possible to initiate transient kinetic experiments with CmlI in nearly a single form at pH 9, simplifying the setup of experiments and the data analysis. The stopped-flow instrument was made anaerobic by being flushed with a dithionite solution followed by anaerobic buffer. CmlI was reduced as described in the [Supporting Information](#) and rapidly mixed with buffer containing varying concentrations of O<sub>2</sub> and substrate using the stopped-flow instrument (see figure legends). In some cases, substrate was added anaerobically to the diferrous CmlI (CmlI<sup>red</sup>) solution before it was mixed with O<sub>2</sub>-containing buffer. In cases in which the P was preformed, it was generated by rapid mixture with an equivalent volume of O<sub>2</sub>-saturated buffer (or in some cases, buffer containing 0.9 equiv of O<sub>2</sub>) in the gastight syringe. In the reactions with NH(OH)-CAM, CmlI was not reduced but was made anaerobic before the reaction was performed.

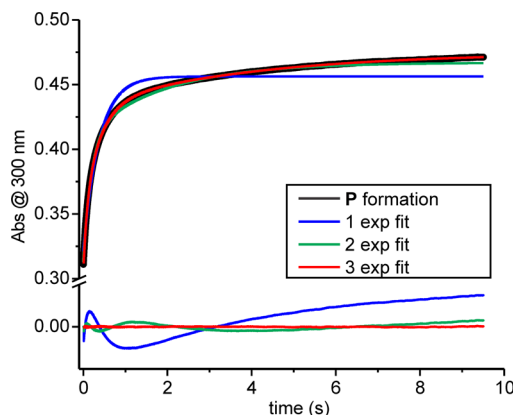
Methods for analyzing reaction kinetics to determine rate constants and or dissociation constants are described in the [Supporting Information](#). These methods include procedures for fitting the time course to multiexponential equations and concentration dependencies to hyperbolic expressions.

**Mössbauer Analysis of the Reaction of CmlI<sup>red</sup> with O<sub>2</sub> and NO-CAM.** Mössbauer samples were prepared using an Update 715 ram syringe controller to mix solutions and dispense the mixtures onto counter-rotating aluminum wheels cooled with liquid nitrogen.<sup>27,28</sup> The time between mixing and freezing was controlled using a calibrated set of delay tubing between the mixer and a nozzle over the wheels. The counter-rotating wheels grind the frozen solution into a powder, which is deposited into the liquid nitrogen bath below the wheels. It is then collected and packed into a Mössbauer cup. In the experiments described here, one syringe held 1.25 mM <sup>57</sup>Fe-enriched CmlI<sup>red</sup> in 50 mM Bicine (pH 9), and the other held O<sub>2</sub>-saturated buffer with 6.25 mM NO-CAM. Data were collected at 20 ms, 80 ms, 250 ms, and 30 s. The Mössbauer spectra were analyzed as described in the [Supporting Information](#).

## RESULTS

**P Formation Reaction.** The P diferrous-peroxo intermediate of CmlI forms in <1 s when chemically reduced diferrous CmlI (CmlI<sup>red</sup>) is combined with O<sub>2</sub>. P is highly stable in the absence of substrate, exhibiting a t<sub>1/2</sub> of ~3 h at 4 °C and pH 9,<sup>2</sup> and thus, the formation reaction is expected to be irreversible. To determine the rate constant(s) for P formation, anaerobic CmlI<sup>red</sup> was mixed with 5–36 equiv of O<sub>2</sub> using a stopped-flow

ultraviolet–visible (UV–vis) instrument. The formation of **P** was followed at 300 nm, the wavelength at which optical feature changes due to **P** formation are maximized (see Figure S1 for the rationalization of wavelength choice here and in reactions described below). The resulting traces are best fit by a three-summed-exponential expression (Figure 2). A complete

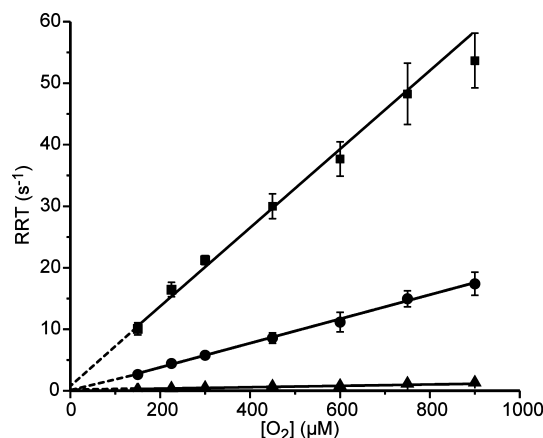


**Figure 2.** Optical changes correlated with the formation of **P** by combining 40  $\mu\text{M}$  CmlI<sup>red</sup> with 450  $\mu\text{M}$  O<sub>2</sub> in buffer (final, postmix concentrations). Single-exponential (blue), double-exponential (green), and triple-exponential (red) fits of the **P** formation process shown by the black trace. Fit residuals are shown below the trace and highlight the need for a three-exponential fit (50 mM Bicine at pH 9 and 4 °C).

description of the techniques used for the analysis of transient kinetic data presented in this study can be found in the [Supporting Information](#). Plotting the reciprocal relaxation times ( $1/\tau$  or RRT) versus O<sub>2</sub> concentration shows that all three phases have a linear dependence on O<sub>2</sub> concentration with apparent second-order rate constants of  $58.1 \pm 2.0$ ,  $19.6 \pm 0.4$ , and  $1.4 \pm 0.1 \text{ s}^{-1} \text{ mM}^{-1}$  (Figure 3). The magnitude of the total amplitude change stays the same over the range of O<sub>2</sub> concentrations tested, and the amplitudes of the three observed phases are approximately equal.

The presence of three phases that are linearly dependent on substrate is most easily ascribed to three parallel reactions to form **P**, rather than a single **P** formation process that employs three consecutive steps, which would yield a nonlinear or no concentration dependence in two of the three phases. The Mössbauer spectrum of **P** shows only one type of cluster.<sup>2</sup> If there are multiple Mössbauer-indistinguishable forms of **P**, they all appear to react with substrates (see below). The multiple-phase **P** formation behavior is likely to be the result of either of the following scenarios or both: (a) three different pathways for O<sub>2</sub> binding that all lead to the same **P** or (b) the existence of CmlI<sup>red</sup> in three different structural or protonation states that differentially affect **P** formation. While the Mössbauer spectra of CmlI<sup>red</sup> show only a single species, the spectra of CmlI<sup>ox</sup> reveal that several forms exist in a pH-dependent distribution.<sup>2</sup> The concentration dependence plots of all phases have a *y*-intercept close to zero, showing that the formation of **P** in the absence of substrate is nearly irreversible, as expected. This finding is also in accord with our previous demonstration that **P** does not exchange with headspace O<sub>2</sub> over the course of hours.<sup>22</sup>

**Kinetic Parameters of the Reaction of **P** with NH<sub>2</sub>-CAM.** The next step of the CmlI cycle that can be isolated is the reaction of **P** with NH<sub>2</sub>-CAM to form NH(OH)-CAM and



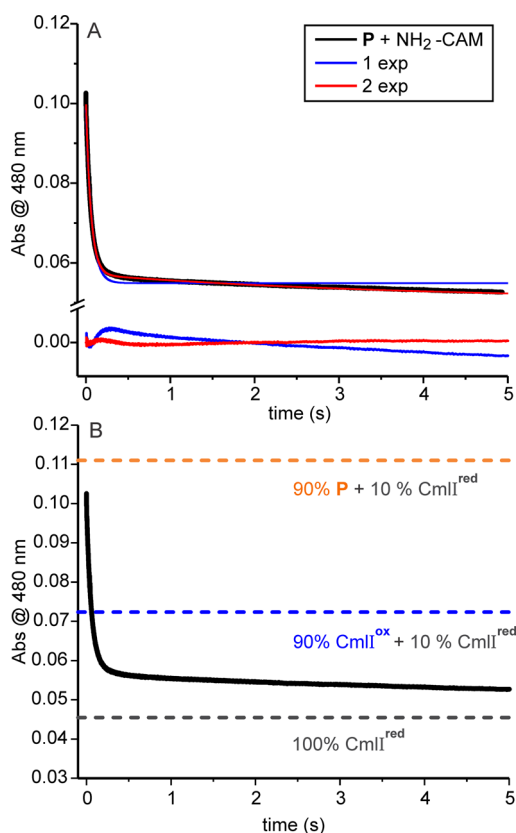
**Figure 3.** O<sub>2</sub> concentration dependence of **P** formation. Forty  $\mu\text{M}$  CmlI<sup>red</sup> (final, postmix concentration) was mixed in a 1:1 ratio with varying concentrations of O<sub>2</sub> in buffer. RRT-1, -2, and -3 (■, ●, and ▲, respectively) from multiexponential fitting of stopped-flow traces are plotted vs O<sub>2</sub> concentration, all showing linear relationships. The second-order rate constant for each process was determined from the slope of the linear fit to the data (—), and they are reported in the text. The reported error of each point is one standard deviation from the mean (50 mM Bicine at pH 9 and 4 °C).

CmlI<sup>ox</sup>, followed by oxidation of NH(OH)-CAM to NO-CAM and reduction of the diferric cluster to form CmlI<sup>red</sup>. To explore this reaction, **P** was formed with 0.9 equiv of O<sub>2</sub> (to ensure no excess O<sub>2</sub> in the reaction) and then loaded immediately on the stopped-flow instrument for reaction with 5–40 equiv of NH<sub>2</sub>-CAM dissolved in anaerobic buffer. When monitored at 480 nm, both **P** decay and the subsequent reduction of the diiron cluster appear as a decrease in absorbance. Both processes are first-order or pseudo-first-order, and the reaction stops after the last step; therefore, summed exponential fitting is appropriate (see the [Supporting Information](#) for additional details).

The time courses are fit well by a two-summed-exponential expression (Figure 4A). Both exponentials have positive amplitudes (a positive amplitude sign correlates with a decrease in absorbance), and the phase with a larger RRT contributes ~85% of the total absorbance change. Because the types of reactions involved are effectively irreversible [O–O bond cleavage and 100% diiron cluster reduction (see below)], it is possible to assign the two RRTs to rate constants for two steps of the reaction. The simplest scenario for the reaction shown in Scheme 1A is to assign one RRT to the rate constant for conversion of **P** to CmlI<sup>ox</sup> and the second to the rate constant for reduction of CmlI<sup>ox</sup> to CmlI<sup>red</sup>. However, analysis of the absorbance changes makes this scenario unlikely.

The expected absorbance due to **P**, CmlI<sup>ox</sup>, and CmlI<sup>red</sup> at 480 nm at various stages of the reaction can be determined using the extinction coefficients of the species (Table S1) and the concentration of CmlI in the experiment (there is negligible absorption due to any of the organic CAM substrates at 480 nm). The starting sample [absorbance = 0.11 (Figure 4B, orange dashed line)] was prepared to have 10% CmlI<sup>red</sup> and 90% **P**. The first step in the proposed mechanistic model is the conversion of **P** and NH<sub>2</sub>-CAM to CmlI<sup>ox</sup> and NH(OH)-CAM. The corresponding predicted absorbance of 90% CmlI<sup>ox</sup> and the remaining 10% CmlI<sup>red</sup> would give an absorbance of 0.072 (Figure 4B, blue dashed line). After re-reduction of the CmlI by NH(OH)-CAM formed *in situ*, the end point pure CmlI<sup>red</sup> spectrum would give an absorbance of 0.046 (Figure 4B, black





**Figure 4.** Optical changes at 480 nm correlated with the reaction of 180  $\mu\text{M}$  P with 2 mM  $\text{NH}_2\text{-CAM}$ . (A) Single-exponential (blue) and double-exponential (red) fits of the substrate-mediated P decay process (black). Fit residuals are shown below the trace. (B) Reaction as in panel A. Dashed lines indicate the calculated absorbance of 180  $\mu\text{M}$  P with 20  $\mu\text{M}$  CmlI<sup>red</sup>, 180  $\mu\text{M}$  CmlI<sup>ox</sup> with 20  $\mu\text{M}$  CmlI<sup>red</sup>, and 200  $\mu\text{M}$  CmlI<sup>red</sup> (50 mM Bicine at pH 9 and 4 °C).

dashed line). Consequently, approximately equal changes in absorbance should result from each step. The observation that the rapid segment of the time course results in an absorbance significantly below the blue dashed line shows that more than just the conversion of P to CmlI<sup>ox</sup> has occurred during the fast phase.

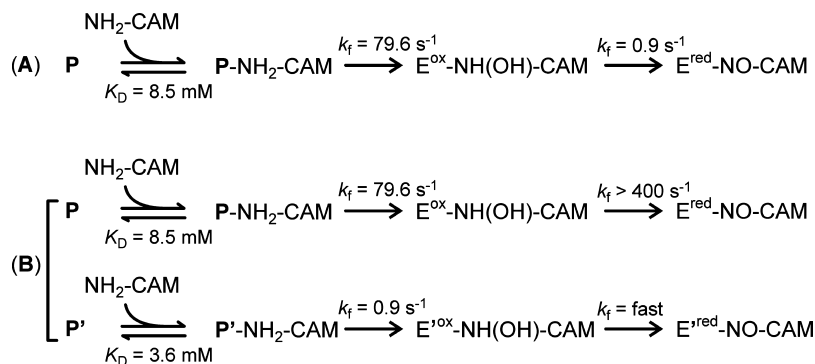
An alternative interpretation for the overall two-phase fit of the time course with unequal amplitudes illustrated in Scheme

1B is that there are two independent processes starting from two different forms of P in an 85:15 ratio (based on the observed ratio of fast and slow phase amplitudes). If the reduction step following the reaction of the dominant form of P with  $\text{NH}_2\text{-CAM}$  is much faster ( $>5$ -fold), the reaction will appear as a single-exponential time course proceeding below the absorbance demarcated by the blue dashed line. Thus, the dominant phase would be reflective of both P decay and fast reduction. The second observed phase would then describe a smaller fraction of P reacting similarly, but with a lower initial rate constant.

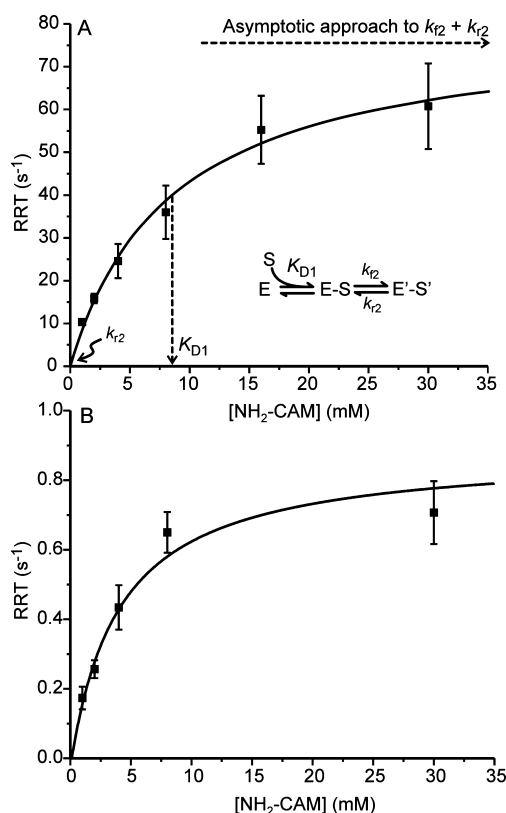
The selection of part B of Scheme 1 over part A is also supported by plots of the RRTs from each exponential phase versus the concentration of  $\text{NH}_2\text{-CAM}$  (Figure 5). The resulting plots are hyperbolic, suggesting that an unobserved, fast, reversible second-order  $\text{NH}_2\text{-CAM}$  binding process precedes P decay (see the Supplemental Experimental Procedures of the Supporting Information). The dissociation constants ( $K_D$ ) for complex formation reactions for the faster and slower processes are  $8.5 \pm 1.5$  and  $3.6 \pm 1$  mM, respectively. Analysis of the hyperbolic curves (see Figure 5A and the Supporting Information) shows that the rate constants for the step following binding are as follows:  $k_f = 79.6 \pm 5 \text{ s}^{-1}$  and  $k_r \approx 0 \text{ s}^{-1}$  for the faster process and  $k_f = 0.9 \pm 0.1 \text{ s}^{-1}$  and  $k_r \approx 0$  for the slower process. The irreversible nature of the reaction is reasonable considering that it involves the cleavage of an O–O bond and insertion of an O atom into an N–H bond as P reacts with  $\text{NH}_2\text{-CAM}$ . If the reaction is irreversible, then in Scheme 1A, the concentration dependence on  $\text{NH}_2\text{-CAM}$  will be lost in the second phase, which is not the case. Moreover, it is shown below that modeling the time course of the complete biosynthetic pathway requires a reduction step with a rate constant that is 2–3 orders of magnitude greater than that shown in Scheme 1A.

**Reaction of CmlI<sup>ox</sup> with  $\text{NH}(\text{OH})\text{-CAM}$ .** The reduction of CmlI<sup>ox</sup> by  $\text{NH}(\text{OH})\text{-CAM}$  can also be monitored by simply mixing these two reactants anaerobically. Careful scrubbing of  $\text{O}_2$  from the reaction buffers and the stopped-flow instrument ensures that the reaction stops after the reduction process and does not proceed to re-form P for the final oxidation step. To study this step, CmlI<sup>ox</sup> was made anaerobic and then mixed in a 1:1 ratio with anaerobic buffer containing varying concentrations of  $\text{NH}(\text{OH})\text{-CAM}$ , all of which were appropriate for establishing pseudo-first-order conditions. The resulting optical

#### Scheme 1. Proposed Schemes for the Reaction of P with $\text{NH}_2\text{-CAM}$ <sup>a</sup>



<sup>a</sup>Rate constants are derived from the plots in Figure 5. For the sake of simplicity, CmlI<sup>ox</sup> and CmlI<sup>red</sup> are abbreviated as E<sup>ox</sup> and E<sup>red</sup>, respectively. P' and E' represent CmlI species that are kinetically distinct from P and E, respectively. Part A represents the case in which there is a single reaction pathway for P reacting with  $\text{NH}_2\text{-CAM}$ . Part B represents the case in which two parallel pathways are possible beginning from two forms of P.

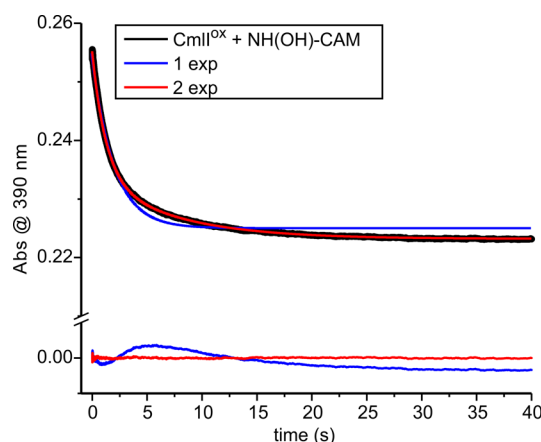


**Figure 5.**  $\text{NH}_2\text{-CAM}$  concentration dependence of the RRTs for the reaction of **P** with  $\text{NH}_2\text{-CAM}$ . Extracted RRTs plotted vs  $[\text{NH}_2\text{-CAM}]$  for the reaction of **P** (180  $\mu\text{M}$ , final postmix concentration) with varying concentrations of  $\text{NH}_2\text{-CAM}$  in anaerobic buffer. The generic method for analysis is illustrated in panel A, where **E** and **E'** are two forms of the enzyme, **S** is a substrate, and **S'** is a modified form of **S** (see the [Supporting Information](#)). (A) For RRT-1, a hyperbolic fit gives the following:  $K_D = 8.5 \pm 1.5$  mM,  $k_f = 79.6 \pm 5$  s $^{-1}$ , and  $k_r \approx 0$  s $^{-1}$ . (B) For RRT-2, a hyperbolic fit gives the following:  $K_D = 3.6 \pm 1$  mM,  $k_f = 0.9 \pm 0.1$  s $^{-1}$ , and  $k_r \approx 0$  s $^{-1}$  (50 mM Bicine at pH 9 and 4 °C).

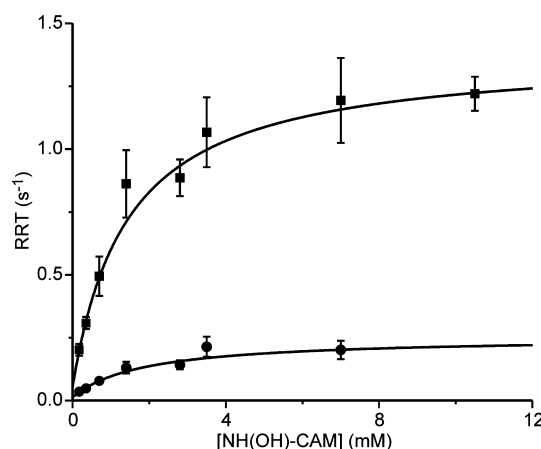
change at 390 nm was a decrease in absorbance; the time course trace fit to a double-summed-exponential equation ([Figure 6](#)). The monitoring wavelength was chosen to maximize the optical change while minimizing the contribution of the  $\text{NH(OH)-CAM}$  that occurs at lower wavelengths. The net absorbance change of  $\sim 0.03$  matched the expected absorbance change for the conversion of 40  $\mu\text{M}$   $\text{CmlI}^{\text{ox}}$  to  $\text{CmlI}^{\text{red}}$ .

The plots of RRT-1 and RRT-2 versus  $\text{NH(OH)-CAM}$  concentration are both hyperbolic ([Figure 7](#)), suggesting that a relatively fast step, presumably  $\text{NH(OH)-CAM}$  binding ( $K_D = 1.1$  mM), precedes the  $\text{CmlI}$  reduction occurring in the observable steps. The rate constants extracted from these plots (for RRT-1,  $k_f = 1.3 \pm 0.1$  s $^{-1}$  and  $k_r \approx 0$ , and for RRT-2,  $k_f = 0.23 \pm 0.03$  s $^{-1}$  and  $k_r \approx 0$ ) are very slow compared with the rate constant for reduction of the diiron cluster observed when starting the reaction with  $\text{NH}_2\text{-CAM}$  as described above. The slower phase disappears as the concentration of  $\text{NH(OH)-CAM}$  increases, suggesting that the biphasic kinetics arise from some sort of allosteric effect of the substrate that will not be pursued further here. A model for the reaction of  $\text{CmlI}^{\text{ox}}$  and  $\text{NH(OH)-CAM}$  is shown in [Scheme 2](#).

The results indicate that the reduction of the diiron cluster by  $\text{NH(OH)-CAM}$  occurs much faster when this pathway

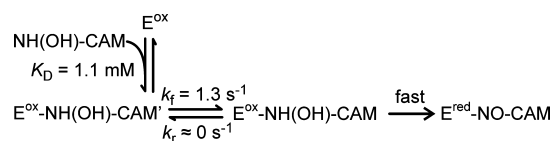


**Figure 6.** Optical change at 390 nm upon the anaerobic mix of 40  $\mu\text{M}$   $\text{CmlI}^{\text{ox}}$  and 2 mM  $\text{NH(OH)-CAM}$  (final, postmix concentrations). The trace fits best to a two-exponential equation, as shown by the colored fit lines and the residuals for one-exponential (blue) and two-exponential (red) fits shown below the trace (50 mM Bicine at pH 9 and 4 °C).



**Figure 7.**  $\text{NH(OH)-CAM}$  concentration dependence of the RRTs for the reaction of 40  $\mu\text{M}$  anaerobic  $\text{CmlI}^{\text{ox}}$  (final, postmix concentration) and varying concentrations of  $\text{NH(OH)-CAM}$  in anaerobic buffer: (■) hyperbolic fits of RRT-1s extracted from the traces of the reaction, giving  $K_D = 1.1 \pm 0.05$  mM,  $k_f = 1.3 \pm 0.1$  s $^{-1}$ , and  $k_r \approx 0$ , and (●) hyperbolic fits of RRT-2s extracted from the traces of the reaction, giving  $K_D = 1.2 \pm 0.3$  mM,  $k_f = 0.23 \pm 0.03$  s $^{-1}$ , and  $k_r \approx 0$ . The amplitude of RRT-2 progressively decreases and is not observed above 8 mM  $\text{NH(OH)-CAM}$  (50 mM Bicine at pH 9 and 4 °C).

## Scheme 2. Proposed Scheme for the Reaction of $\text{CmlI}^{\text{ox}}$ with $\text{NH(OH)-CAM}$ <sup>a</sup>



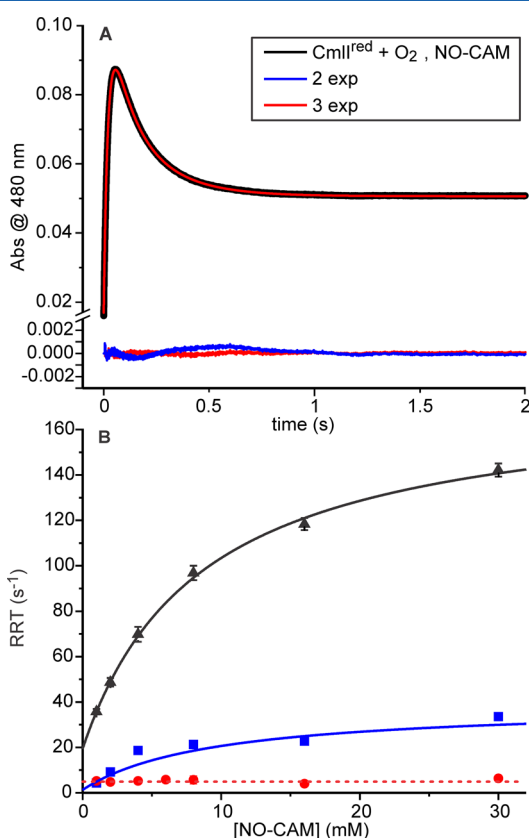
<sup>a</sup>For the sake of simplicity,  $\text{CmlI}^{\text{ox}}$  and  $\text{CmlI}^{\text{red}}$  are abbreviated as  $E^{\text{ox}}$  and  $E^{\text{red}}$ , respectively. The intermediate  $E^{\text{ox}}\text{-NH(OH)-CAM}$  and the assignment of the rate constant for the reduction step as “fast” are proposed on the basis of modeling of the overall biosynthetic reaction pathway kinetics (see [Simulation of the Overall Reaction](#)).

intermediate is generated *in situ* in the active site rather than having to bind from solution. Moreover, if  $\text{NH(OH)-CAM}$  dissociates from the active site after it is generated, but before it

can reduce the diiron cluster, the subsequent reduction step would presumably occur with the slow rate constants observed for the reaction of  $\text{NH}(\text{OH})\text{-CAM}$  in solution with  $\text{CmlI}^{\text{ox}}$ . This route for cluster reduction should thus be considered an off-pathway process.

**Reaction of P with NO-CAM.** Anaerobic P was formed as described above and mixed with NO-CAM dissolved in anaerobic buffer. Surprisingly, little to no reaction was observed when the mixture was monitored at 480 nm (Figure S2). Alternative ways to approach this reaction allow the kinetic time course to be monitored and the observation of product as described below.

**Reaction of  $\text{CmlI}^{\text{red}}$  with NO-CAM and  $\text{O}_2$ .** The reaction in which NO-CAM is converted to CAM can be examined by mixing  $\text{CmlI}^{\text{red}}$  with a buffer solution containing a 5–150-fold excess of NO-CAM and a 5-fold excess of  $\text{O}_2$  (a saturated solution) (Figure 8A). At 480 nm, one would expect to observe an increase in absorbance as P is formed and then a decrease as the NO-CAM is converted to CAM with the formation of

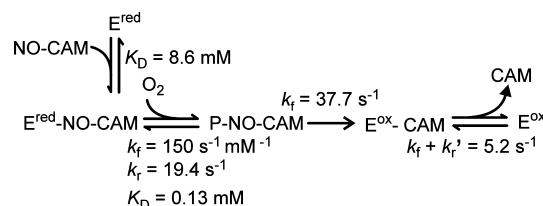


**Figure 8.** Reaction of  $\text{CmlI}^{\text{red}}$  with NO-CAM in  $\text{O}_2$ -saturated buffer. (A) Optical changes at 480 nm upon mixing  $200 \mu\text{M}$   $\text{CmlI}^{\text{red}}$  with buffer containing 2 mM NO-CAM and 0.9 mM  $\text{O}_2$  (all postmix, final concentrations). A three-exponential fit (red) best simulates the time course and is shown superimposed on the data (black). Residuals for two-exponential (blue) and three-exponential (red) fits are shown below the data. (B) Fits of the RRTs vs NO-CAM concentration. For the RRT-1 hyperbolic fit (black),  $k_f = 150.4 \pm 8.8 \text{ s}^{-1} \text{ mM}^{-1}$  (at 0.9 mM  $\text{O}_2$ ),  $k_r = 19.4 \pm 2 \text{ s}^{-1}$ ,  $K_{\text{D-binding}} = 8.6 \pm 1 \text{ mM}$ , and  $K_{\text{D-observed step}} = 0.13 \text{ mM}$ . For the RRT-2 hyperbolic fit (blue),  $k_f = 37.7 \pm 5 \text{ s}^{-1}$  and  $k_r \sim 0 \text{ s}^{-1}$ . For the RRT-3 linear fit (red), an estimate of the concentration-independent rate constant ( $k_f + k_r'$ ) is  $5.2 \pm 0.1 \text{ s}^{-1}$ . The error ranges for the blue and red points are within the size of the symbol (50 mM Bicine at pH 9 and 4 °C).

$\text{CmlI}^{\text{ox}}$ . Conducting the reaction in this way gave time courses that are fit well by a three-summed-exponential equation. The resulting RRTs were plotted versus NO-CAM concentration as shown in Figure 8B. The faster two phases show a hyperbolic dependence on NO-CAM concentration, while the slowest phase shows no dependence. In this case, the amplitudes of the two faster phases have opposite signs, making it unlikely that they arise from parallel reactions. The hyperbolic dependencies indicate that there is a fast, reversible step that precedes the observable reaction, which must itself consist of at least three additional steps in accord with the three-summed-exponential fit.

Although the observed kinetics of the reaction of  $\text{CmlI}^{\text{red}}$  with  $\text{O}_2$  and NO-CAM would support a variety of four-step processes that include a binding step, Scheme 3 shows one

### Scheme 3. Proposed Scheme for the Reaction of $\text{CmlI}^{\text{red}}$ with NO-CAM<sup>a</sup>

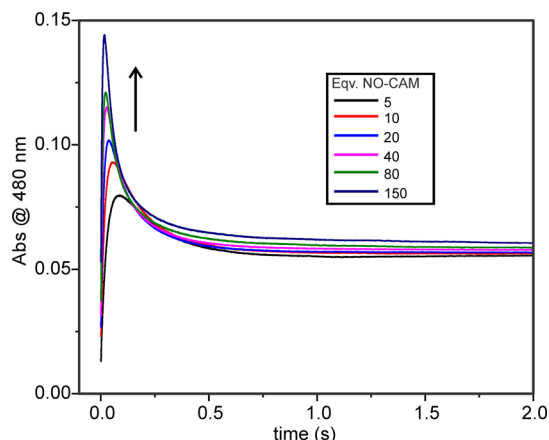


<sup>a</sup>For the sake of simplicity,  $\text{CmlI}^{\text{ox}}$  and  $\text{CmlI}^{\text{red}}$  are abbreviated as  $E^{\text{ox}}$  and  $E^{\text{red}}$ , respectively.

possibility that is reasonable based on the chemistry of the biosynthetic pathway. In this model, NO-CAM binding is the fast reversible step that precedes the optically observable steps with a  $K_{\text{D}}$  of  $8.6 \pm 1 \text{ mM}$ . Reversible (Figure 8B, black curve, non-zero y-intercept)  $\text{O}_2$  binding occurs in the following step to form P, giving a fast increase in absorbance. The reversible nature of this step would be expected to yield a nonlinear plot for another RRT, as observed. In the model in Scheme 3, the P reacts in the subsequent step with NO-CAM irreversibly to give a decrease in absorbance. The presence of two reversible steps in the three-step series to this point (beginning with binding) should, in principle, be analyzed using the roots of a cubic equation. However, the large difference in the observed maximum RRTs allows the rate constants for the individual steps to be approximated from the maximum ( $k_f + k_r'$ ) and intercept ( $k_r$ ) of the two hyperbolic plots. This approximation gives  $k_f = 150.4 \text{ s}^{-1} \text{ mM}^{-1}$  (at 0.9 mM  $\text{O}_2$ ) and  $k_r = 19.4 \text{ s}^{-1}$  for  $\text{O}_2$  binding ( $K_{\text{D}} = 0.13 \text{ mM}$ ) to form P and  $k_f = 37.7 \text{ s}^{-1}$  and  $k_r = 0$  for the reaction of P with bound NO-CAM. The irreversible nature of the latter step (zero intercept for the plot in Figure 8B, blue curve) uncouples the third RRTs from the NO-CAM concentration, so the final concentration-independent step occurs with  $k_f + k_r' = 5.2 \text{ s}^{-1}$  (Figure 8B, red curve). This step is likely to be product release and to represent the rate constant limiting substrate flux through the cycle. The small amplitude of this slow phase (<10% of those of the faster phases) is consistent with the very small change in absorbance at 480 nm caused by the binding of CAM to  $\text{CmlI}^{\text{ox}}$ .<sup>22</sup>

At high NO-CAM concentrations, the faster RRT reaches values that far exceed the expected values based on the rate constant for P formation in the absence of NO-CAM (see Figure 3). This suggests that in the NO-CAM complex,  $\text{O}_2$  binding is facilitated, allowing much faster P formation. It also

suggests that NO-CAM binding precedes O<sub>2</sub> binding, consistent with the increasing maximum value of **P** formed as the concentration of NO-CAM increases (Figure 9). Any **P** that



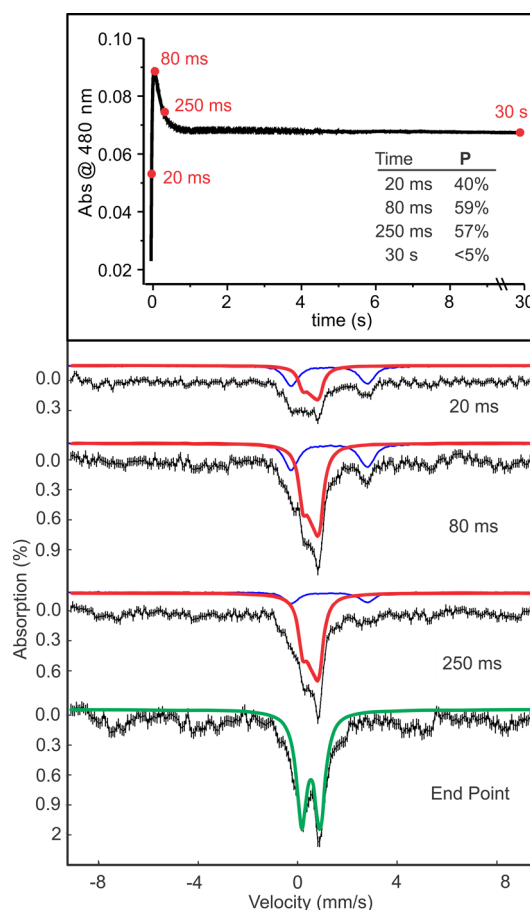
**Figure 9.** NO-CAM concentration dependence of the time course of the reaction of CmlI<sup>red</sup> with pseudo-first-order concentrations of O<sub>2</sub> and NO-CAM. The traces follow the reaction of 200 μM CmlI<sup>red</sup> with 1–30 mM NO-CAM in O<sub>2</sub>-saturated buffer (50 mM Bicine at pH 9 and 4 °C).

forms prior to NO-CAM binding would yield a dead-end complex, as the results presented above and in Figure S2 show that **P** does not react with NO-CAM. Because there is no substantial buildup of **P** at the end of the reaction, it is likely that NO-CAM binding is very fast.

**Verification of **P** as the Active Oxidant That Acts on NO-CAM.** One possible explanation for the failure of preformed **P** to react with NO-CAM is that a different form of **P** is made when NO-CAM is present in the active site. To examine this possibility, short time point samples of the reaction of CmlI<sup>red</sup> with an excess of NO-CAM and O<sub>2</sub> were prepared by a rapid-freeze quench procedure and analyzed by Mössbauer spectroscopy (Figure 10, top).

Mössbauer analysis (Figure 10, bottom) showed a decrease in the level of CmlI<sup>red</sup> over the course of the reaction, concomitant with an increase in the level of **P**. The spectral parameters for **P** formed in this way are the same as those for **P** formed in the absence of substrate (Table S2).<sup>2</sup> The level of **P** increases to a maximum and decays following approximately the time course of the 480 nm species observed in the reaction of NO-CAM with CmlI<sup>red</sup> and O<sub>2</sub> (Figure 9). At a long time (30 s), <5% **P** is present and the sample is predominantly diferric. Previous experiments have shown that CAM is formed in this reaction.<sup>22</sup> Thus, Mössbauer analysis suggests that intermediate **P** formed in the presence or absence of substrate has identical Mössbauer parameters and is the oxidant that acts on NO-CAM prebound in the active site.

**Kinetic Parameters of the Complete Reaction Pathway.** The complete biosynthetic pathway was examined in two ways: (a) by pre-equilibrating NH<sub>2</sub>-CAM anaerobically with CmlI<sup>red</sup> to allow the complex to reach equilibrium and then initiating the reaction by mixing with O<sub>2</sub>-saturated buffer or (b) by mixing CmlI<sup>red</sup> with O<sub>2</sub>-saturated buffer containing various concentrations of NH<sub>2</sub>-CAM. No significant difference in trends or magnitudes of RRTs was observed between the two iterations. The data presented are for the latter combination. All optical changes were observed by stopped-flow UV–vis spectroscopy at 480 nm, 4 °C, and pH 9. This wavelength

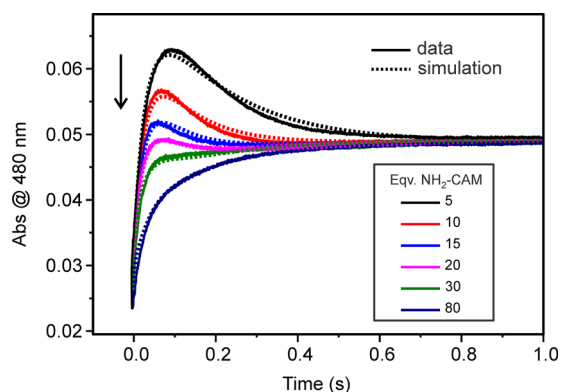


**Figure 10.** Time points (top) of RFQ samples for Mössbauer analysis of the reaction of 200 μM CmlI<sup>red</sup> with 0.9 mM O<sub>2</sub> and 2 mM NO-CAM (final, postmix concentrations). The inset table shows the fractions of the sample present as **P** quantified from the Mössbauer spectra. Mössbauer spectra of the samples (bottom). The black lines are the experimental data, and the colored lines are fits to various species: diferrous (blue), intermediate **P** (red), and resting state diferric (green). A small fraction of mononuclear ferric ion also appears at the end point (50 mM Bicine at pH 9 and 4 °C).

was chosen because it gives the maximum optical change for the **P** formation and **P** decay processes. Using a shorter wavelength gives a greater optical change for **P** formation, but **P** decay is masked by the reappearance of the Fe–O–Fe charge transfer band of diferric CmlI, which has a shoulder at ~375 nm. Initiating the reaction in this way allows both **P** formation and substrate-mediated **P** decay to be observed (Figure 11). After **P** forms and decays, the resulting CmlI<sup>ox</sup> is reduced by the NH(OH)-CAM product of the first reaction to give a further decrease in absorbance. This reduction is followed by binding of O<sub>2</sub> to re-form **P**, resulting in an increase in absorbance. Finally, **P** decays as CAM is formed to give a decrease in absorbance.

In sharp contrast to the trend shown above for the reaction of CmlI<sup>red</sup> with O<sub>2</sub> and NO-CAM, the maximum amount of **P** formed decreases with an increasing substrate concentration when NH<sub>2</sub>-CAM is the substrate. This suggests that the two reactions proceed differently. The traces in Figure 11 show that as the concentration of NH<sub>2</sub>-CAM increases, the pseudo-first-order rate constants for **P** formation and decay converge. Unfortunately, this convergence makes the resolution of the RRTs by summed-exponential fitting unreliable.





**Figure 11.**  $\text{NH}_2\text{-CAM}$  concentration dependence of the overall reaction time course. The solid curves are for optical changes at 480 nm from the reaction of  $\text{CmlI}^{\text{red}}$  ( $200\ \mu\text{M}$ ) with 1–16 mM  $\text{NH}_2\text{-CAM}$  (final, postmix concentrations) in  $\text{O}_2$ -saturated buffer and the dashed curves are for simulation of the same reaction using numerical integration. The rate constants and reactions used for the simulation are shown in Scheme 4 (see Discussion). The postmix  $\text{O}_2$  concentration is  $\sim 0.9\ \text{mM}$  at  $4\ ^\circ\text{C}$  (50 mM Bicine at pH 9 and  $4\ ^\circ\text{C}$ ).

Some insight into this complex overall reaction and the differences in **P** reactivity at different steps can be gained by combining the measured kinetic and thermodynamic parameters for each step with numerical integration of the overall reaction. This approach to the analysis is explored in Discussion.

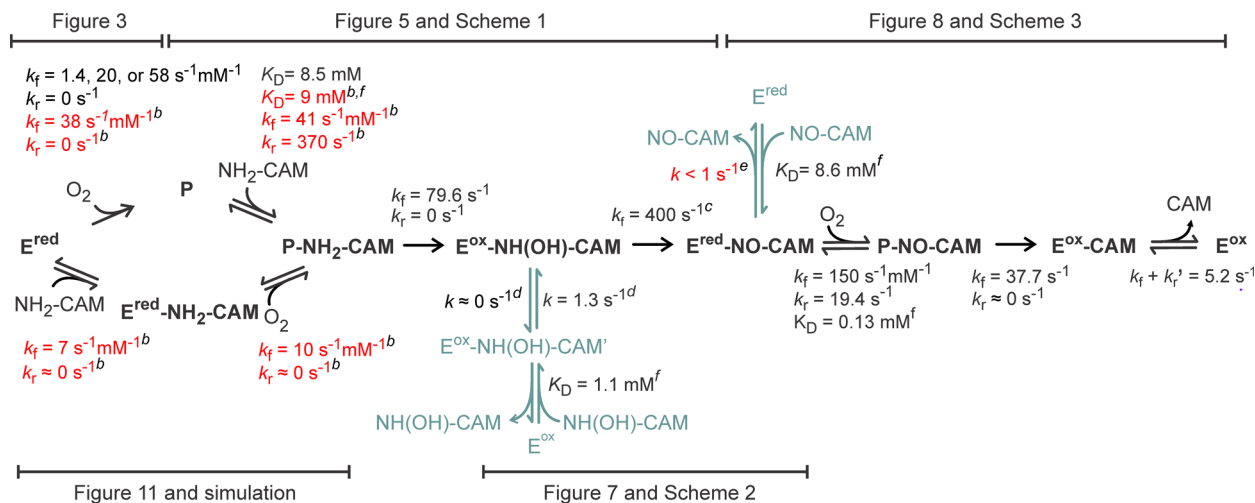
## DISCUSSION

The results presented here show that the complex reaction pathway for the final steps in the biosynthesis of chloramphenicol can be studied in segments to extract rate constants and/

or thermodynamic parameters for each step. The major tenets of the proposed reaction cycle shown in Figure 1 are supported by these studies. In particular, the data demonstrate the oxygenase reactivity of peroxo intermediate **P** with  $\text{NH}_2\text{-CAM}$  and  $\text{NO-CAM}$  as well as the ability of the pathway intermediate  $\text{NH(OH)-CAM}$  to reduce the  $\text{CmlI}$  diferric cluster in preparation for the proposed midpathway re-formation of **P**. The results also reveal several new aspects of the pathway and its regulation, including insight into the effect of substrate binding on the rate constant for  $\text{O}_2$  binding and the dramatic difference in the rate constant for cluster reduction by  $\text{NH(OH)-CAM}$  formed *in situ* versus that binding from solution. Kinetic evidence is found for progress along the biosynthetic pathway at rates that are high compared with those for dissociation of intermediates from the enzyme, providing insight into how the enzyme efficiently accelerates catalysis while maintaining specificity. These new aspects of  $\text{CmlI}$  catalysis are discussed here.

**Regulation of the **P** Formation Rate and Substrate Binding Order.** The set of time courses shown in Figure 11 represents the overall biosynthetic process beginning with  $\text{NH}_2\text{-CAM}$  and  $\text{O}_2$  binding to  $\text{CmlI}^{\text{red}}$ . The time courses look similar when  $\text{NH}_2\text{-CAM}$  is preincubated with  $\text{CmlI}^{\text{red}}$  before being mixed with  $\text{O}_2$ . It is evident that this process differs in two respects from that later in the pathway in which  $\text{CmlI}^{\text{red}}$  reacts with  $\text{NO-CAM}$  and  $\text{O}_2$ . First, the  $\text{NO-CAM}$  reaction has a strict binding order such that preincubation with  $\text{O}_2$  to form **P** stops the reaction (Figure S2). Second, the maximal amount of **P** formed and the apparent rate at which it is formed increase with an increase in  $\text{NO-CAM}$  concentration (Figure 9) but decrease with an increase in  $\text{NH}_2\text{-CAM}$  concentration (Figure 11). This observation is readily rationalized in the case of  $\text{NO-CAM}$  because the  $\text{CmlI}^{\text{red}}\text{-NO-CAM}$  complex must form first

**Scheme 4.** Proposed Scheme for the Overall Reaction of  $\text{CmlI}^{\text{red}}$  with  $\text{NH}_2\text{-CAM}$  and  $\text{O}_2$  To Form  $\text{CAM}^{\text{a}}$



<sup>a</sup>The proposed scheme synthesizes all the kinetic data collected in this study. The figure or scheme that first introduced each rate constant or  $K_D$  value is indicated. For the sake of simplicity,  $\text{CmlI}^{\text{ox}}$  and  $\text{CmlI}^{\text{red}}$  are abbreviated as  $\text{E}^{\text{ox}}$  and  $\text{E}^{\text{red}}$ , respectively. Bold black intermediate names and arrows denote components of the main pathway. Blue-green intermediate names and arrows denote experimentally accessible components but are thought to be side pathways. The rate constants for intermediates leaving by the side pathways are slow and have little effect on the observed time course of the reaction. The rate constants and dissociation constants shown in black were determined experimentally in this work and are fixed in the numerical integration shown in Figure 11 (dashed curves). The rate constants and dissociation constants shown in red were determined by the numerical integration program to best fit the data over a range of substrate concentrations. <sup>b</sup>These constants can only be varied over an only small (>5%) range and still give a reasonable fit to the data. <sup>c</sup>This constant can assume any value of  $>300\ \text{s}^{-1}$ . <sup>d</sup>These constants can be varied over a large range and still give a reasonable fit to the data. <sup>e</sup>The rate of dissociation of  $\text{NO-CAM}$  for this step must be  $<1\ \text{s}^{-1}$ . <sup>f</sup>The  $K_D$  refers to the binding reaction.



to allow **P** formation in a way that can go on to form CAM (Figure S2). In contrast, the rationale for the decreasing level of accumulation of **P** in the NH<sub>2</sub>-CAM reaction proposed in Scheme 4 is more complex. The lack of a preferred order of addition of substrates in this case suggests that it can proceed via two parallel routes. Experimental data are reported here for the rate constants and binding  $K_D$  values for the route in which O<sub>2</sub> can bind first (constants colored black in Scheme 4) (Figures 3 and 5). The results show that in the absence of NH<sub>2</sub>-CAM, **P** forms by three kinetically distinguishable routes. However, as shown by the numerical integration fit to the data shown in Figure 11 (see below), when even low levels of NH<sub>2</sub>-CAM [or NO-CAM] (see Figure 8 and Scheme 3) are present, only one rate constant for **P** formation is required to fit the time course. Interestingly, the observed rate constant is not the same as any found in the absence of a substrate. This finding suggests that the substrate makes the CmlI<sup>red</sup> population homogeneous with respect to **P** formation while altering the rate of the process, by a mechanism that is currently not understood. The dissociation and rate constants for the branch of the pathway starting with NH<sub>2</sub>-CAM binding are more difficult to determine directly because of the lack of a spectral change upon NH<sub>2</sub>-CAM binding, the similarity of the rate constants for **P** formation and decay, and the inability to limit the reaction to a few steps. However, the presence of this alternative pathway is necessary to account for the decreasing maximal level of accumulation of **P** with an increasing NH<sub>2</sub>-CAM concentration. This observation is true because, at low concentrations of NH<sub>2</sub>-CAM, the initial O<sub>2</sub> binding pathway is preferred and **P** decays slowly after it is formed because of the relatively low affinity of **P** for NH<sub>2</sub>-CAM ( $K_D = 8.5$  mM). Consequently, **P** maximally accumulates. As the NH<sub>2</sub>-CAM concentration increases, the rapid, high-affinity NH<sub>2</sub>-CAM binding pathway is preferred, leading efficiently to the **P**-NH<sub>2</sub>-CAM intermediate, which is very rapidly converted to CmlI<sup>ox</sup>-NH(OH)-CAM. Consequently, neither **P** nor the complex of **P** with a substrate accumulates.

**In Situ versus Bimolecular Reactions.** After CmlI<sup>ox</sup>-NH(OH)-CAM is formed in either the reaction pathway beginning with CmlI<sup>red</sup> reacting with O<sub>2</sub> and NH<sub>2</sub>-CAM or that beginning with **P** reacting with NH<sub>2</sub>-CAM, a very rapid reduction occurs to form CmlI<sup>red</sup>-NO-CAM. It is likely that both of these reaction pathways involve *in situ* reduction of the diiron cluster by NH(OH)-CAM before it can escape the active site. The best supporting evidence of this proposal is the very slow reduction observed when the reaction is started by mixing CmlI<sup>ox</sup> with NH(OH)-CAM. This latter reaction proceeds with a fast, reversible binding reaction followed by slow reduction. It is possible that before reduction can occur, NH(OH)-CAM must reorient from the position of initial binding [CmlI<sup>ox</sup>-NH(OH)-CAM'] to the position adjacent to the diiron cluster that it would occupy if it were formed *in situ* [CmlI<sup>ox</sup>-NH(OH)-CAM]. We speculate that this reorientation is the slow step, so that the reduction reaction itself remains very fast as required by the overall flux through the reaction pathway (see below). The net effect of *in situ* reduction without intermediate dissociation is an 80-fold increase in flux through this step as well as a guarantee that most of the NH(OH)-CAM formed will not be lost from the pathway or undergo adventitious reactions.

A similar *in situ* versus biomolecular scenario appears to apply to the following steps in the pathway in which CmlI<sup>red</sup>-NO-CAM is converted to **P**-NO-CAM and then CmlI<sup>ox</sup>-CAM.

Our results show that the bimolecular reaction in which CmlI<sup>red</sup> binds NO-CAM and then reacts with O<sub>2</sub> is a viable reaction. However, if NO-CAM is generated *in situ* by starting with the NH<sub>2</sub>-CAM reaction, the dissociation of NO-CAM before O<sub>2</sub> binding would lead to an inefficient reaction. This observation is true because unbound NO-CAM generated in this way would be at a very low concentration compared with the concentration used to study the reaction of CmlI<sup>red</sup> with NO-CAM in O<sub>2</sub>-saturated buffer (Figure 8). Consequently, the fast O<sub>2</sub> binding reaction to CmlI<sup>red</sup> would lead to the irreversible formation of **P**, which cannot react with NO-CAM not already bound to the enzyme (Figure S2). Our previously published results show that labeled NO-CAM can exchange from the diferrous site in a single-turnover reaction initiated by reacting **P** with NH<sub>2</sub>-CAM.<sup>22</sup> However, in this case, labeled NO-CAM is at a high concentration in solution. Even so, the exchange is inefficient, and approximately half of the NO-CAM does not exchange.

**Simulation of the Overall Reaction.** Using the assumptions of a dual pathway for the starting NH<sub>2</sub>-CAM oxidation and limited exchange for the NH(OH)-CAM and NO-CAM intermediates, the overall mechanism shown in Scheme 4 was evaluated versus the experimental time course using numerical integration. To limit overparametrization of the simulation, all of the rate constants and most of the  $K_D$  values determined experimentally in this study were fixed (black values), leaving only a few others as variable (red values). The numerical integration program adjusted these latter values to achieve an acceptable fit as shown by the dashed curves in Figure 11. In steps in which only  $K_D$  values were determined or in which multiple rate constants were found experimentally, values were also allowed to float. In these cases, the best fit values (red) were consistent with the experimental approximations (black). Very few of the fit values on the main biosynthetic pathway could be altered by >5% and still give an acceptable fit (see notes for Scheme 4). However, the rate constants for the side pathways shown in blue in Scheme 4 could be varied over a much larger range without affecting the fit so long as they did not exceed specific limits. At their maximum values, these constrained rate constants were all very slow in comparison with the rate constants found in the main biosynthetic pathway.

The excellent fit to the data obtained supports the basic tenets of Scheme 4. In particular, the dual pathway for the starting NH<sub>2</sub>-CAM oxidation does accurately model the decreasing yield of **P** as the concentration of NH<sub>2</sub>-CAM increases, the *in situ* NH(OH)-CAM-mediated reduction reaction must be very fast, and slow steps, such as those found in the reaction of CmlI<sup>ox</sup> reduced by direct mixing with NH(OH)-CAM, cannot be part of the main biosynthetic pathway.

It is shown by the data in Figure 3, as well as our past investigations, that **P** formation is effectively irreversible in the absence of substrate.<sup>22</sup> The simulation in Scheme 4 indicates that it is also nearly irreversible in the presence of NH<sub>2</sub>-CAM but becomes reversible when NO-CAM is bound. The interaction of substrate and O<sub>2</sub> binding in the active site is also evident from the order dependence of NO-CAM and O<sub>2</sub> binding in the reaction with CmlI<sup>red</sup>, as noted above. It seems likely that this steric or electronic interaction affects the stability of the **P** intermediate. Indeed, our recently published model for the structure of **P** places the distal oxygen of the peroxo moiety immediately adjacent to the nitrogen substituent of the bound

aromatic substrate.<sup>23</sup> It remains unclear whether all substrates affect **P** stability, because the kinetics of the reaction pathway may dictate whether the reversibility is actually observed. For example, the very fast steps following **P**-NH<sub>2</sub>-CAM formation may mask a slow rate of release of O<sub>2</sub> from this species.

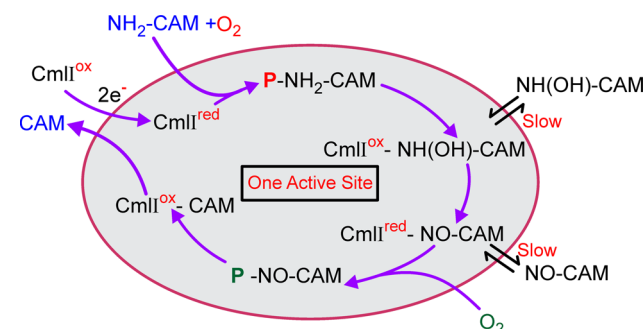
**Comparison of the Biosynthetic Pathways of CmlI and AurF.** CmlI and AurF share a very high degree of sequence similarity, and they have nearly identical structures in the vicinity of the active site. The N-oxygenation reactions that they catalyze are analogous, although AurF has a smaller natural substrate, *p*-aminobenzoate versus NH<sub>2</sub>-CAM. Nevertheless, detailed mechanistic studies of both enzymes support a key difference in their biosynthetic pathway involving the identity of the pathway intermediate responsible for the internal reduction step.<sup>19,22</sup> Where we have proposed that NH(OH)-CAM fills this role in the CmlI mechanism, Bollinger, Krebs, and co-workers have proposed that dihydroxylamino-benzoate serves as the reductant.<sup>19</sup> The latter species is proposed to be formed from the preceding hydroxylamino-benzoate intermediate by reaction with the AurF peroxo species equivalent to **P**. One would usually assume that two biological systems with this level of similarity would use the same mechanism. However, our study suggests that, in this case, both mechanisms may be correct. The results presented here indicate that CmlI is designed to largely retain the pathway intermediates in the active site. If the retention of intermediates is not as stringent in the case of AurF, much more of the analogous hydroxylamino-benzoate may dissociate. Once released, it could then react with the AurF peroxo intermediate to yield the dihydroxylamino-benzoate or a similar intermediate. Indeed, if NH(OH)-CAM is reacted anaerobically with CmlI **P**, a reaction similar to that reported for AurF occurs, resulting in CAM and CmlI<sup>red</sup>.<sup>22</sup> Thus, a delicate balance between the rate of intermediate dissociation and the rate of reduction may control the course of the reaction chemistry even in nearly identical enzymes. The CmlI mechanism is controlled by the fact that the reduction step occurs at a rate faster than the rate of NH(OH)-CAM dissociation. The enzyme system uses kinetic control to drive the reaction forward with very high efficiency and specificity.

## CONCLUSION

CmlI catalyzes a rare biochemical reaction that must overcome at least three mechanistic hurdles. (a) Three individual two-electron oxidation reactions must be catalyzed by the same enzyme and involve (as shown here) the same reaction cycle reactive species, **P**. (b) The inherent reactivity of the resulting biosynthetic pathway intermediates must be controlled to avoid adventitious reactions and loss of the eventual product. (c) The reactive intermediate of the reaction cycle must perform oxidative chemistry with the various oxidation states of aromatic amines without adventitious oxidation of other moieties such as C–H bonds. CmlI has apparently solved some or all of these mechanistic problems by stabilizing a peroxo rather than a high-valence reactive species, thereby limiting the range of substrates that can be oxidized. This solution required the evolution of a new, more reactive, type of peroxo intermediate, **P**. We have described **P** as being ambiphilic, because a peroxo species can be either nucleophilic or electrophilic as required to match the electron donating capacity of the range of pathway intermediates.<sup>22,23</sup> On the other hand, the peroxo intermediate is not expected to have the extreme oxidizing potential of high-valence oxo species

common to enzymes that catalyze C–H oxidation. The unusual characteristic of CmlI that emerges from this study is its ability to avoid the release of pathway intermediates from the first oxidation step through the release of CAM as illustrated in Scheme 5. This protects the cell from the reactive pathway intermediates and ensures both specificity and efficiency in antibiotic production.

**Scheme 5. Model for the CmlI Biosynthetic Pathway in One Active Site**



## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00695.

Supplemental Experimental Procedures, a list of extinction coefficients (Table S1), and supplemental results (Figures S1 and S2) (PDF)

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### Notes

The authors declare no competing financial interest.

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